1. Supplemental figures and legends

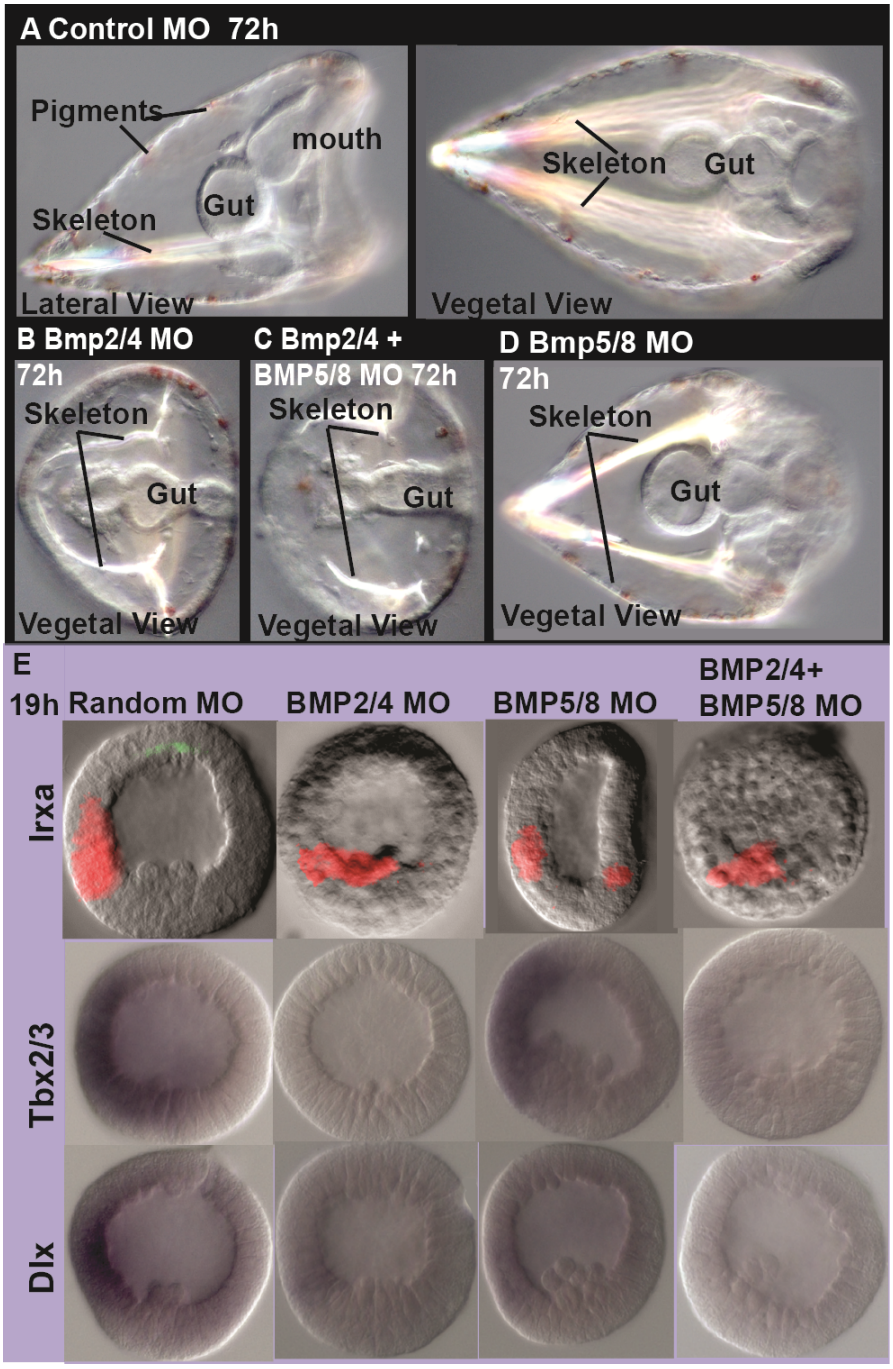


Figure S1. Phenotypes of the inactivation of the BMP2/4 and BMP5/8 ligands. A-D, phenotypes of the inactivation of the BMP2/4 and BMP5/8 ligands at 72h. A, sea urchin embryo injected with Control MO that does not affect normal development. Left – lateral view, right – vegetal view. B, embryo injected with BMP2/4 MO. C, Co-injection pf BMP2/4 and BMP5/8 MO. D, Embryo injected with BMP5/8 MO. E. Effect of BMP2/4 and BMP5/8 MO on the spatial expression pattern of tbx2/3, irxa and dlx at 19h. BMP2/4 MO reduces the expression of tbx2/3, irxa and dlx, but the expression of irxa at the ectoderm–endoderm border remains. BMP5/8 MO has weaker effect on the genes expression. Co-injection of BMP2/4 and BMP5/8 MO almost eliminates the expression of tbx2/3 and dlx, while still not affecting the vegetal ectoderm expression of irxa.

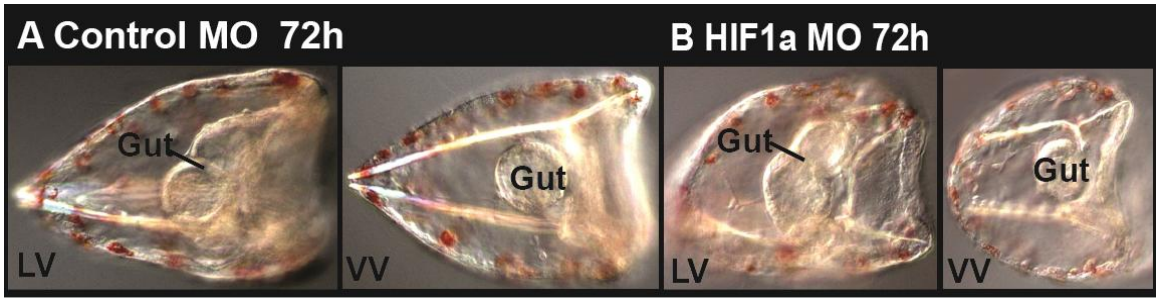


Figure S2. Phenotypes of the inactivation of the transcription factor HIF1α. A, sea urchin embryo injected with Control MO that does not affect normal development, 72h. B, embryo injected with HIF1α MO shows delayed development and a smaller shape.

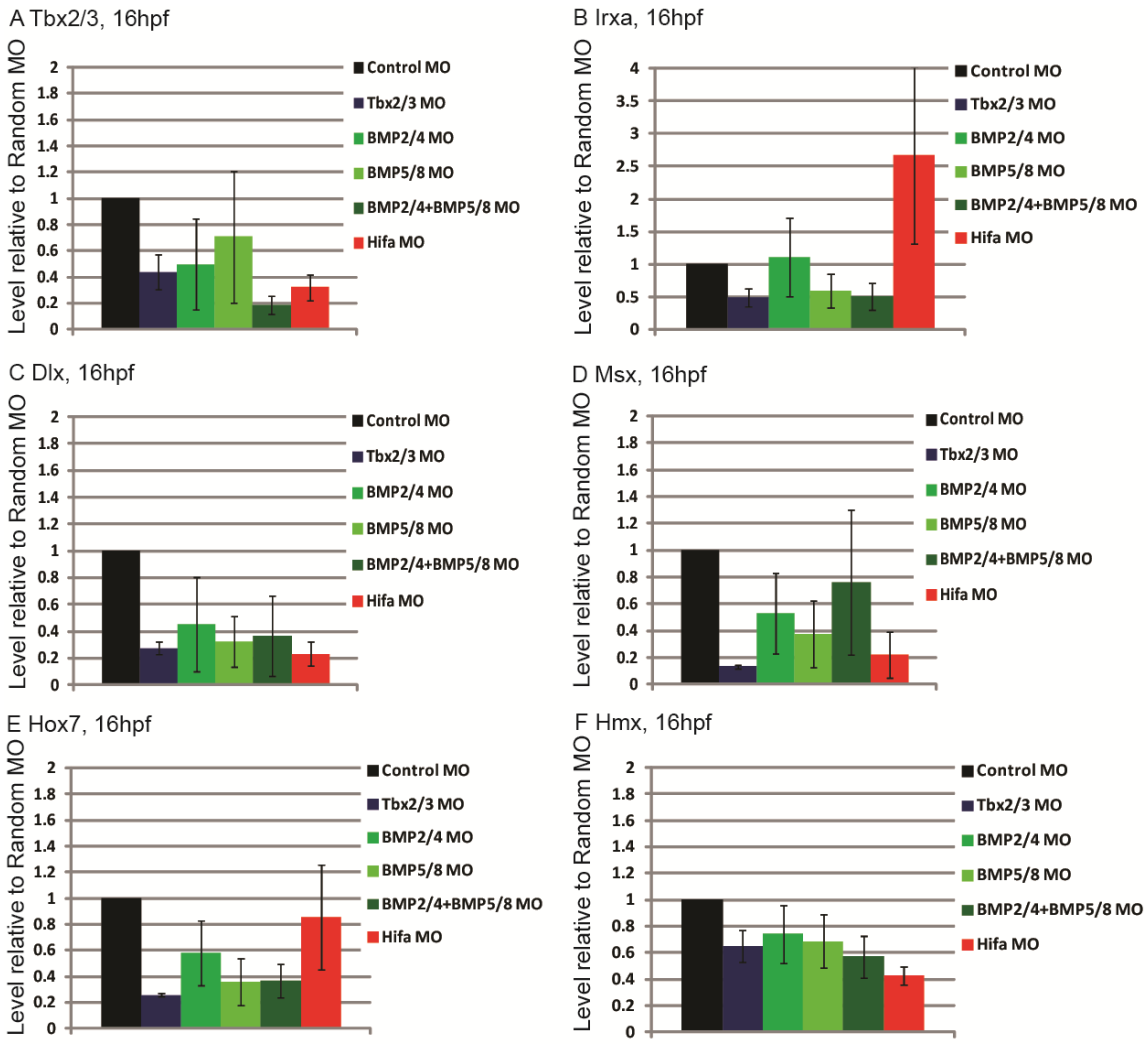


Figure S3. Perturbation analysis statistics at 16h. Shown is the average ratio between the expression level of a gene in a specific MO injection versus its level in control MO injection, measured by QPCR. (Tbx2/3, Dlx and Msx n=3, Hmx, Irxa and Hox7 n=4, error bars show standard error).

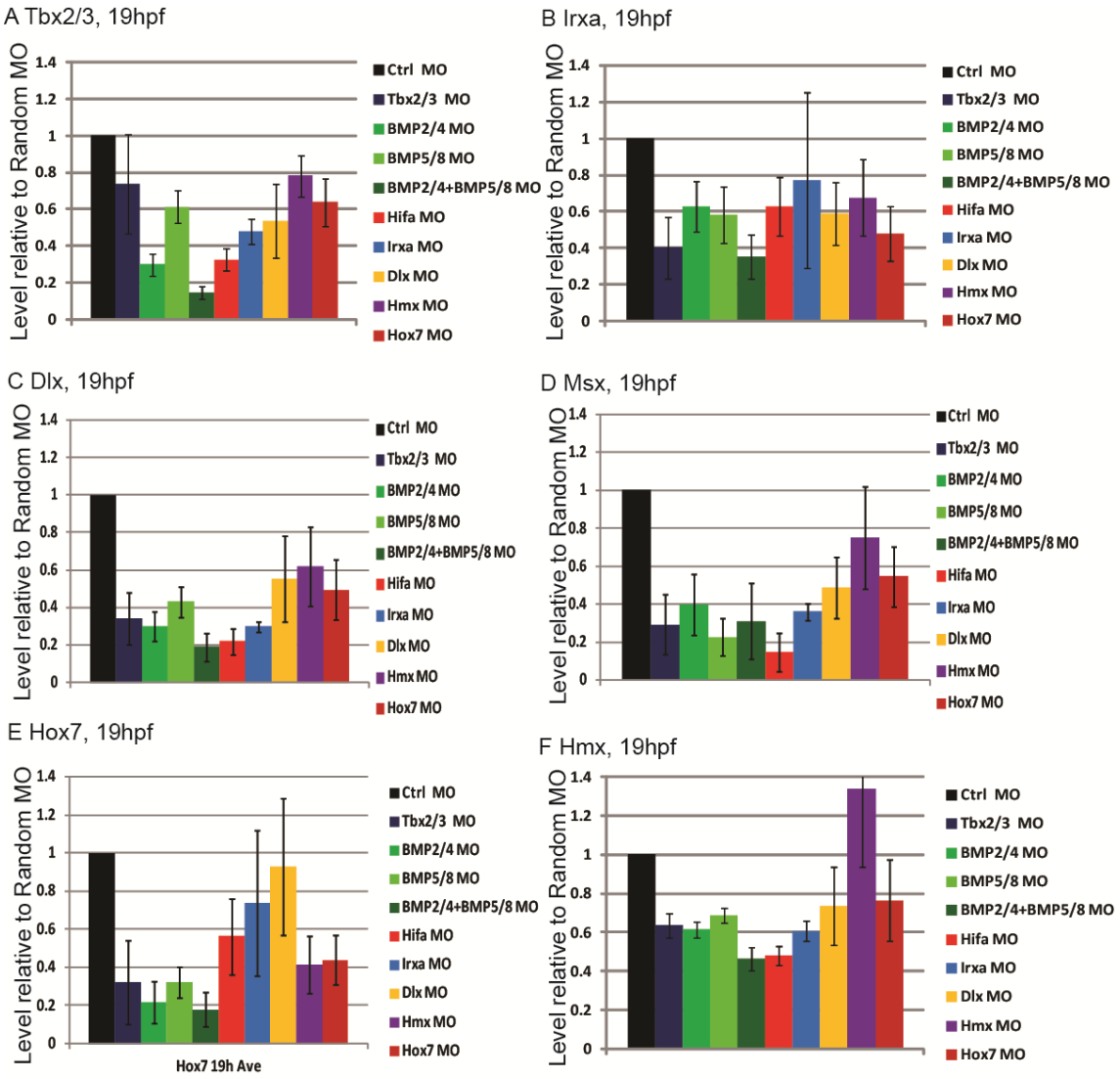


Figure S4. Perturbation analysis statistics at 19h. Shown is the average ratio between the expression level of a gene in a specific MO injection versus its level in control MO injection, measured by QPCR. (Tbx2/3 MO n=7, BMP2/4 MO, BMP5/8 MO and BMP2/4+BMP5/8 MO n=5, HIF1α MO n=5, Irxa MO, Dlx MO and Hox7 MO n=4, Hmx MO n=3. Error bars show standard error).

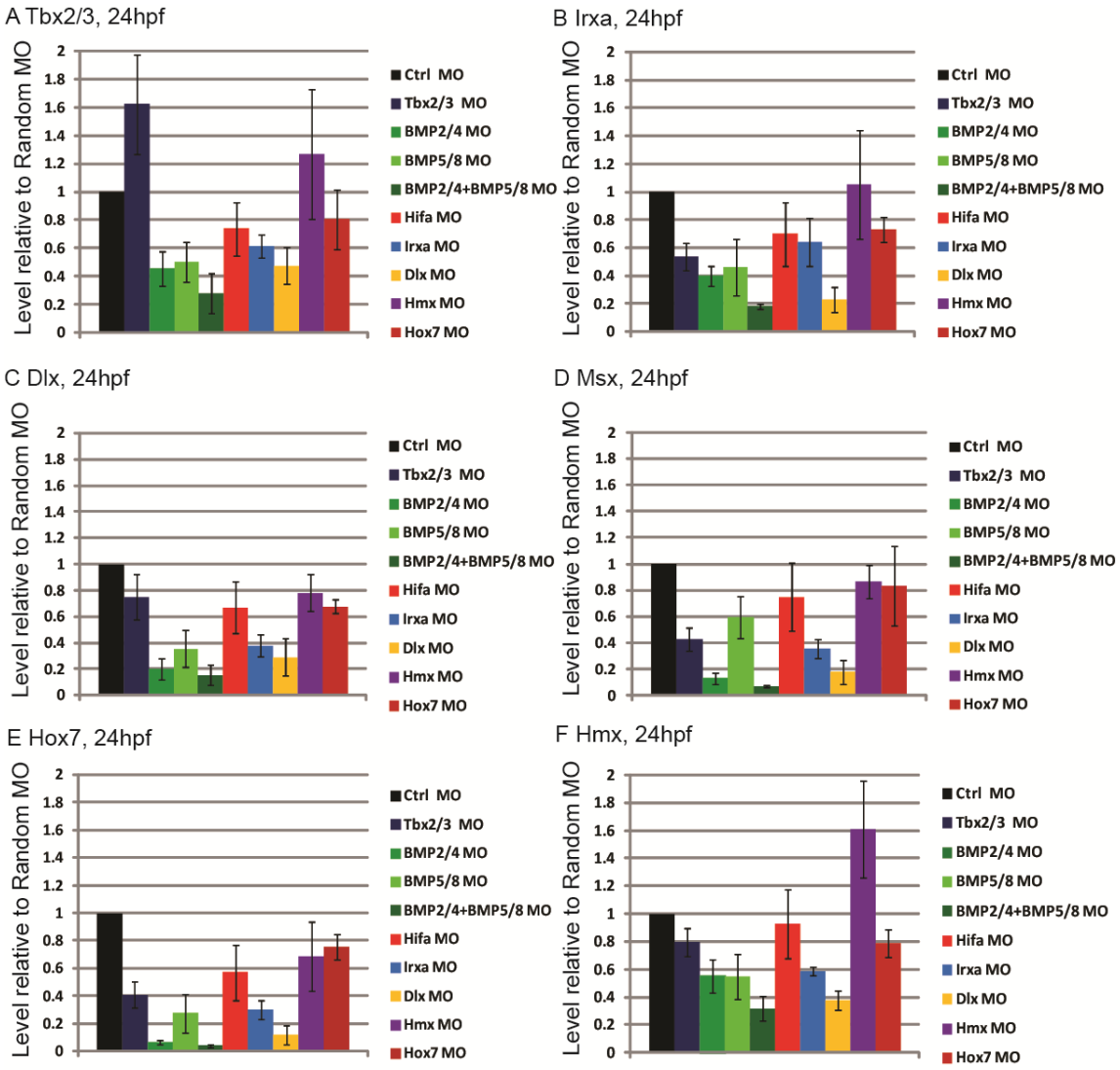
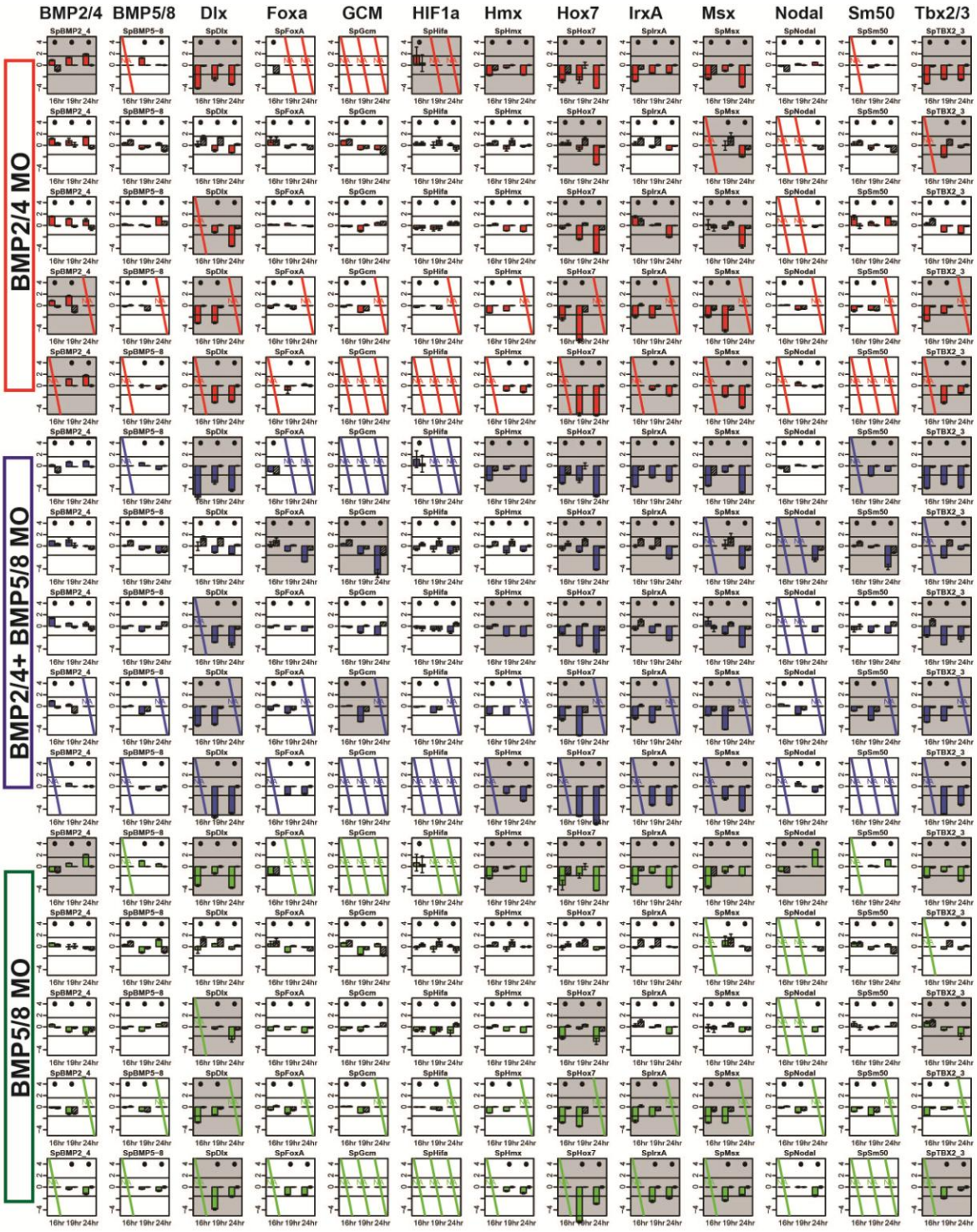
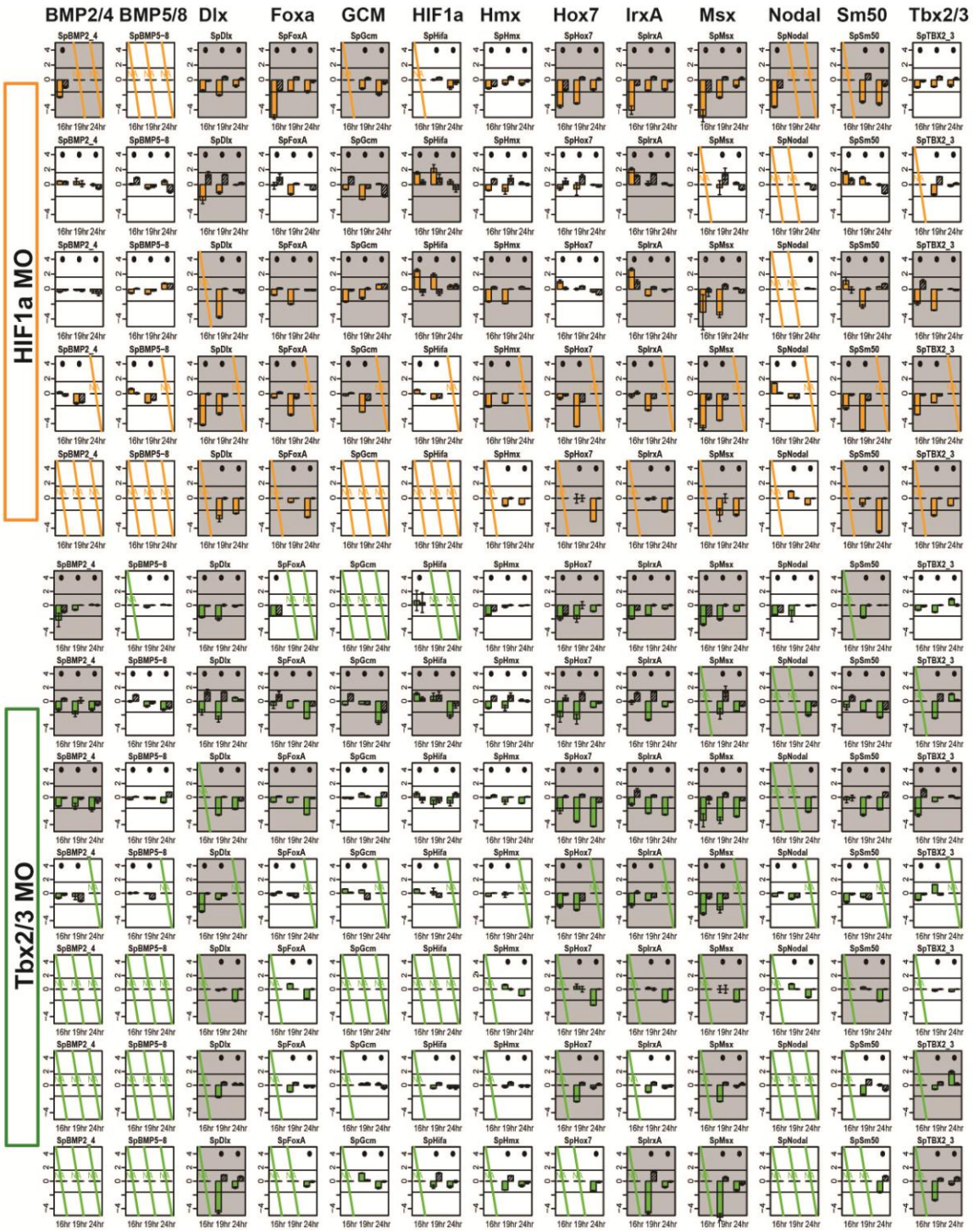


Figure S5. Perturbation analysis statistics at 24h. Shown is the average ratio between the expression level of a gene in a specific MO injection versus its level in control MO injection, measured by QPCR. (Tbx2/3 MO n=6, BMP2/4 MO, BMP5/8 MO and BMP2/4+BMP5/8 MO n=4, HIF1α MO n=4, Irxa MO, and Hox7 MO n=4, Dlx MO and Hmx MO n=3. Error bars show standard error).


BMP2/4
BMP5/8
Dlx
Foxa
GCM
HIF1a
Hmx
Hox7
IrxA
Msx
Nodal
Sm50
Tbx2/3
BMP2/4 MO
BMP2/4+ BMP5/8 MO
BMP5/8 MO
16hr 19hr 24hr

S6A.


BMP2/4
BMP5/8
Dlx
Foxa
GCM
HIF1a
Hmx
Hox7
IrxA
Msx
Nodal
Sm50
Tbx2/3
HIF1a MO
Tbx2/3 MO
16hr 19hr 24hr

S6B

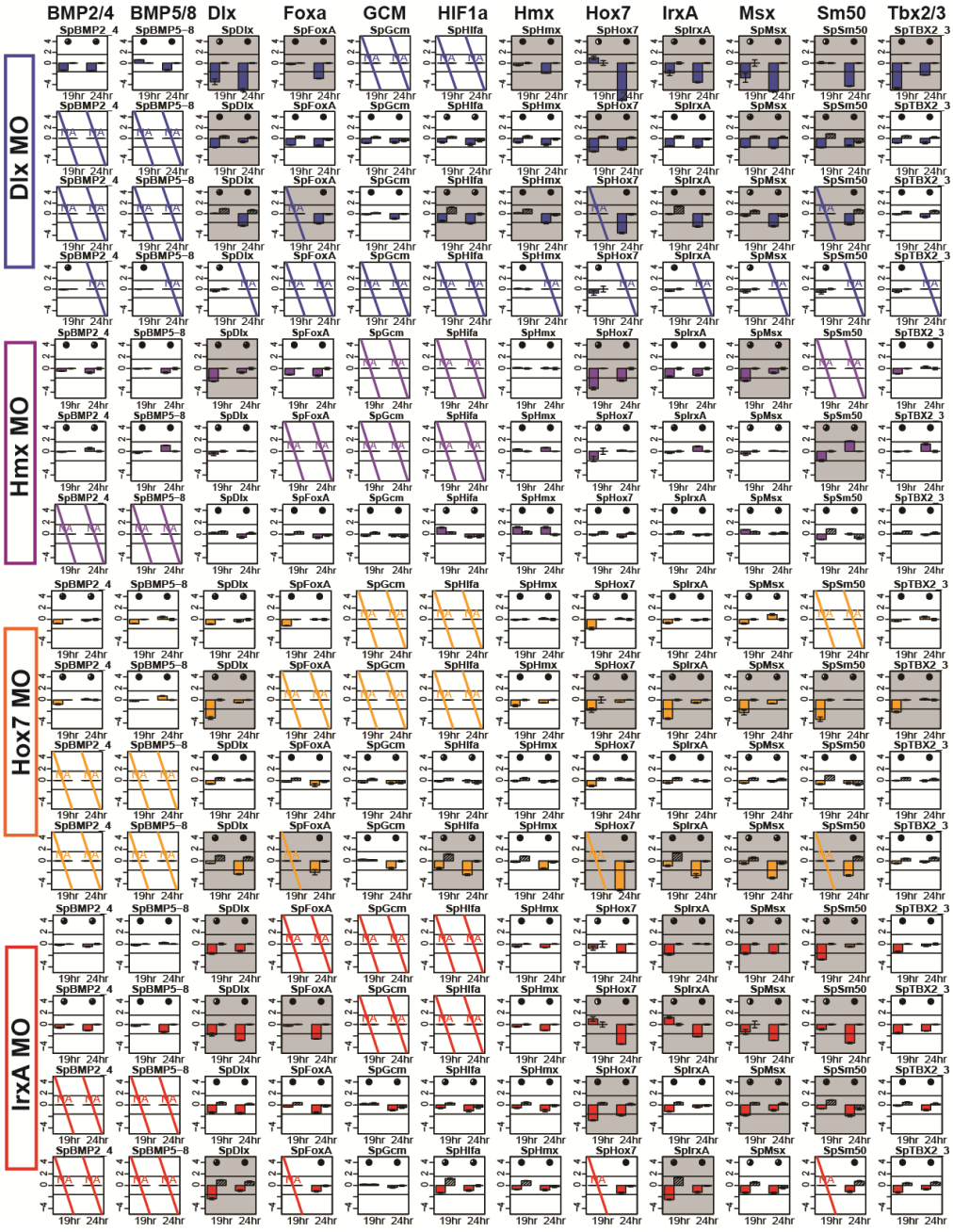


Figure S6. Quantitative perturbation data: A, B - BMP2/4, BMP5/8, Tbx2/3 and HIF1α MO's at16h, 19h, 24h. C. Dlx, Hmx, Hox7 and Irxa MO's at19h, 24h. Changes in gene expression levels were measured by QPCR and plotted as the change in cycle number. Shown is the difference between normalized gene expression levels in embryos treated with specific MO and levels in embryos injected with control MO, at 16hpf, 19hpf and 24hpf. The gene specific MO used for each experiment is given in the row headings at left. The response of embryos to the gene specific MO compared to control MO is shown by colored boxes. The response of the embryos to control MO compared to un-injected embryos is shown by slashed rectangles. Each data point reflects the average of three technical replicates. Error bars are standard errors. Grey plot backgrounds indicate cycle number changes that are greater than 1.6 (more than threefold increase in the expression level) or lower than -1.6 (more than threefold decrease of expression levels).

S6C.

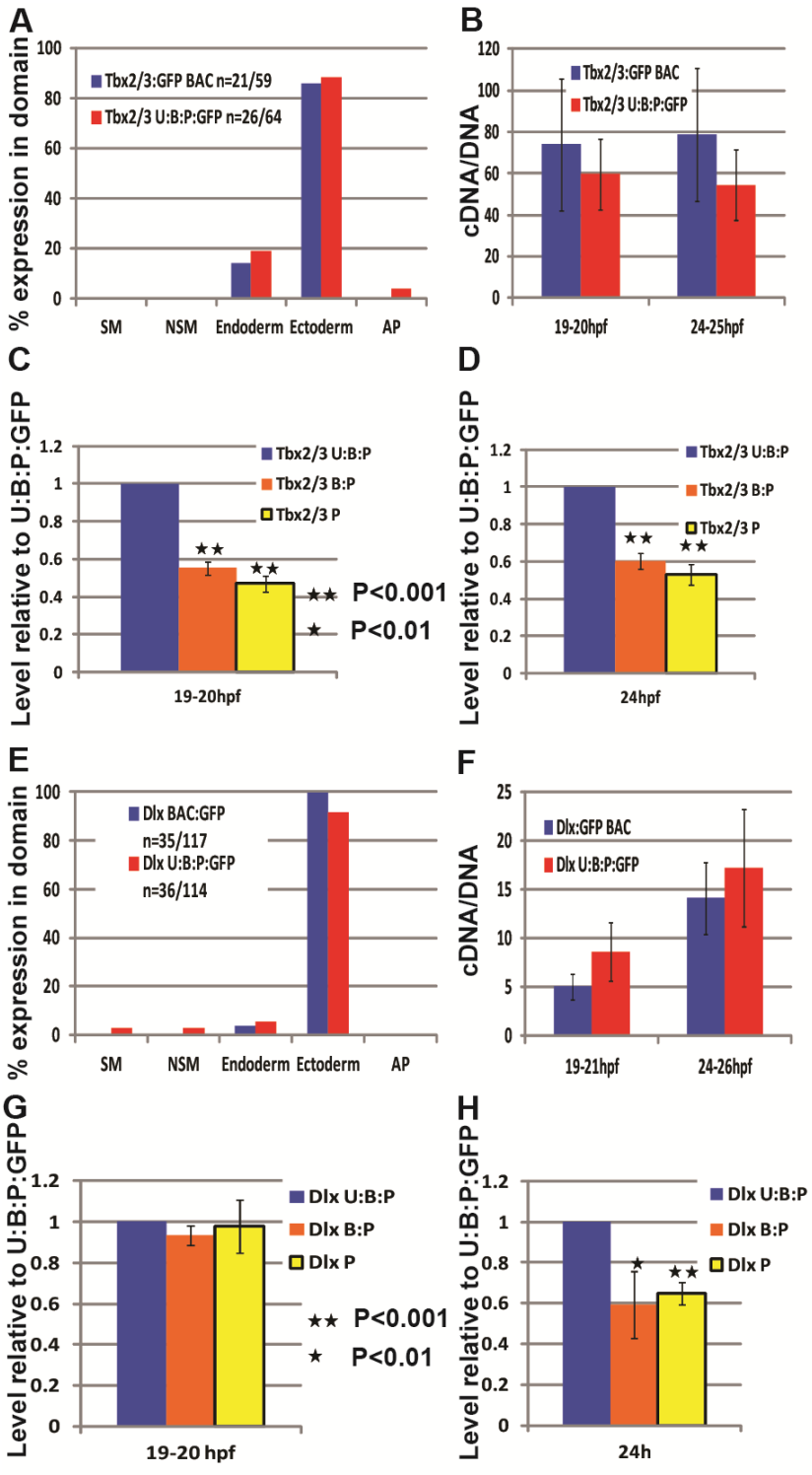


Figure S7. Cis-regulatory analysis of tbx2/3 and dlx. A. Expression of the SpTbx2/3 GFP recombinant BAC and of U:B:P:GFP reporter both show similarity to the endogenous gene expression at the aboral ectoderm and endoderm at 24hpf. B. Similar expression levels of the SpTbx2/3 GFP recombinant BAC and the U:B:P:GFP reporter indicate that U:B:P:GFP contains sufficient regulatory information for the correct expression of tbx2/3. C, D, Deletion analysis of tbx2/3 U:B:P:GFP construct at two time points. E. Expression of the SpDlx GFP recombinant BAC and of U:B:P:GFP reporter both show similarity to the endogenous gene expression at the aboral ectoderm at 24hpf. F. Similar expression levels of the SpDlx GFP recombinant BAC and the U:B:P:GFP reporter indicate that U:B:P:GFP contains sufficient regulatory information for the correct expression of Dlx. G, H, Deletion analysis of Dlx U:B:P:GFP construct at two time points.

2. Supplemental tables

Table S1: MO and QPCR primer list.

Gene	MO	QPCR Primers
BMP2/4	ACCCCAATGTGAGGTGGTAACCAT	F: CCAGCAAGGTCGAAGAACTC R: CTCTACCCGACGACGATGAT
BMP5/8	AAGATAAATGCGGGAGCACGAACAT	F: CAGGATACCCCAAGGTGAGA R: CTACCTCGTTCCCTCAGCAG
Dlx	CAACGTCAAACGAATACATCAAAGC	F: CCAGCTTACAACTCCAACAGC R: TTACCTGAGTTTGAGTGAGTCCA
HIF-1α	GGTCGCCATAATCGGTCTCTGAATC	F: CGATCACGAAGAGGGAAAGA R: TGGGAAGCACTTTTGAAACC
Hmx	GATAGTTCACGGCTACTGTCCATAG	F: TCGTCGTTTGAAGGTTGAAGT R: TGATAGACGCATCTTGCTCG
Hox7	GACGAAATACGAACTAGAACTCATG	F: GGCAGACTTACACCCGCTAC R: TCTGTCGTTCTGTCAATCCG
Irxa	GTTTTTTCTTTCTGAACTTACCTGA	F: TATGGAATGGACCTGAACGG R: TATGATCTTTTCGCCCTTGG
Msx	TGCACGTCGATTCGATAGAAGAAAA	F: AGCACAAGACAAACCGGAAG R: CGCTCGGCTATCGAGAGGTA
Tbx2/3	TTCGATGCCGGTTTCATAGAGAAAG	F: ACTGCCGGTACAAGTTCCAC R: GACACATTTCTGCATCCATTG
Foxa	-	F: CAATAGTGCCATGATCTCGC R: CATGCTGGTGATCGCACTCC
GCM	-	F: CGACTGATAACCACGCTCAA R: TTAACGACGTCGGTCGATTC
Sm50	-	F: TAGCCTTTGCTACGGGTCAA R: CTGAGGCGACGAAACTGAA

Table S2: Binding site mutations

Transcription factor	Intact site	Mutated site
1st and 2nd SMAD sites in Tbx2/3 U	GGTCTCGTCTG	GATATCATATT
3rd and 4th SMAD sites in Tbx2/3 U	AGACTTGTCTG	ATATTTATATT
HIF-1α site in Tbx2/3 U	CACGC	CGTTC
1st SMAD site in Dlx U	GTCTTG	ATATTG
2nd SMAD site in Dlx U	TGTCT	GGGCT
1st Tbx2/3 site in Dlx U	TAACAC	GCATAC
2nd Tbx2/3 site in Dlx U	GGGTGA	GCATAC
HIF-1α 1st site in Dlx U	GCGTG	GCATA
HIF-1α 2nd site in Dlx U	CACGT	TATGT

Table S2 presents the point mutations that were generated for the different transcription factor binding sites in each *cis*-regulatory module. Undelined are matches with the consensus sequences. SMAD consensus: YYAGACR (Uniprobe data set), HIF-1α consensus: RCGTG (Wenger, Stiehl and Camenisch, STKE 2005), Tbx2/3 consensus: GGTGTGA (Wilson and Conlon, Genome. Biol. 2002) and GTGTGA, GGGTGA, or GTGTTA (Carreira et al. Mol. Cell. Biol. 1998), reviewed in (Abrahams, Parker and Prince, IUBMB Life, 2010).

Table. S3: Mutation analysis results and statistics

Mutation/Perturbation	Time	# of Repeats	Average ratio to WT	Standard Error	p-value in z-test
Tbx2/3 U:B:P ΔSMAD×4	19-20hpf	4	0.81	12.5%	0.07
Tbx2/3 U:B:P ΔSMAD×4	**24hpf**	**5**	**0.56**	**7%**	**$<10^{-6}$**
Tbx2/3 U:B:P + BMP2/4 MO	19-20 hpf	3	0.64	17%	0.02
Tbx2/3 U:B:P +BMP2/4 MO	**24 hpf**	**3**	**0.38**	**4%**	**$<10^{-6}$**
Tbx2/3 U:B:P ΔHIF-1α	**19-20hpf**	**4**	**0.68**	**3%**	**$<10^{-6}$**
Tbx2/3 U:B:P ΔHIF-1α	**24hpf**	**5**	**0.74**	**5%**	**2×10^{-6}**
Tbx2/3 U:B:P + HIF-1α MO	**19-20 hpf**	**2**	**0.79**	**1%**	**$<10^{-6}$**
Tbx2/3 U:B:P + HIF-1α MO	**24 hpf**	**2**	**0.78**	**7%**	**0.002**
Dlx U:B:P ΔSMAD×2	19-20hpf	6	0.89	13%	0.033
Dlx U:B:P ΔSMAD×2	**24hpf**	**6**	**0.72**	**3%**	**$<10^{-6}$**
Dlx U:B:P + BMP2/4 + BMP5/8 MO	**19-20 hpf**	**2**	**0.68**	**10%**	**0.0004**
Dlx U:B:P + BMP2/4 + BMP5/8 MO	**24 hpf**	**2**	**0.52**	**4%**	**$<10^{-6}$**
Dlx U:B:P ΔHIF-1α	**19-20hpf**	**3**	**0.82**	**2%**	**$<10^{-6}$**
Dlx U:B:P ΔHIF-1α	24hpf	3	1.08	4%	0.03
Dlx U:B:P + HIF-1α MO	**19-20 hpf**	**2**	**0.81**	**5%**	**6×10^{-5}**
Dlx U:B:P + HIF-1α MO	24 hpf	2	0.79	9%	0.015
Dlx U:B:P ΔTbx2/3×2	19-20hpf	6	0.99	8%	0.45
Dlx U:B:P ΔTbx2/3×2	**24hpf**	**5**	**0.72**	**6%**	**7×10^{-6}**
Dlx U:B:P + Tbx2/3 MO	19-20 hpf	3	0.79	39%	0.29
Dlx U:B:P + Tbx2/3 MO	**24 hpf**	**3**	**0.44**	**10%**	**$<10^{-6}$**

1. Table S3 presents the results and statistics of the mutation and perturbation analysis that were conducted in this work. Marked in bold are the effects we consider to be significantly different than the control ($p<0.01$, one tailed z-test). In Fig. 5 we indicate $p<0.01$ with one star and $p<0.001$ with two stars. In case of mutations the control is the expression level of the wild type construct. In case of perturbation the control is the expression level of the reporter construct coinjected with random MO.

3. Supplemental data: *tbx2/3 and dlx cis*-regulatory elements and the binding sites within them

(A) **Tbx2/3** cis-regulatory element *U* is located -3637 to -2879 upstream of *tbx2/3* translation start and contains four SMAD functional binding site highlighted in green and one HIF-1α functional site highlighted in red. (B) **Tbx2/3** proximal region, *P*, is located -482 upstream of the *tbx2/3* translation start site and down to the translation start site. The 5' UTR region is in italic font. (C) **Dlx** cis-regulatory element U is located -1260 upstream of the start of translation site and down to the start of translation. Is contains two function SMAD sites highlighted in green, two HIF-1α sites highlighted in red and two function Tbx2/3 sites highlighted in blue. The 5' UTR region is in italic font.

A. Tbx2/3 cis-regulatory element U

CAATTCCATCAGAGGTGACGAAAAATTAACTAATAAATTATTTGAAGTTTCCCTGTGTGGTCTCGTCT
GATCAATATTCCTCGGGGTCAGTTTTGTTACCCATTTCTTGATGTCAATGTTCGGCATTTAGTTTGATC
CGATGTCCACTATTCACTGTGTTAGTATCCCGAGATTAATGTAAATAATGTGCCGACACCCTTCCCCA
CTGTGAAAACACTGGTTACAATTTTGACAGCCAACCTTTTCTTTTCTCAATCACCCTTTTCCTTCCCCTT
ACAAAGTGCAATTCCAGAACGATTGGGGCAGTTTAGGGGTGTGAAAAGAGGCGGTTTGGTGGCTG
GAAAAGCTCTATTGAAATCAGAATGGGTTCTTAAAAAACACACGTATAGCGCCATAAGCCGTGCTAC
GATCAGCACCAATTACCTCTATGTTATACTCTCTCTACAGTTATCCCTCGGCTCCGCGCGACACGCCG
GCGACACGAAGCTTTCCCCGATCGAAATTCTATTACACCTCTAAAAAAAGGGGGTGGAAAAAAAGA
GAATAGCTAAAGCTCCGCACCACTTCTTTTGAGAACAAATCGCCCCGATTGTTGGTGTAAAATAGCC
CACTGAAATGAAGTGACGAGGCGATGAAAAGACTTGTCTGCTAGGCAGCTTGAAACAATGTGGATT
AGAATCGGAGTTAGGTGAGAGGGGGGTGCTTTCGAGCAATTCCTCTACTCCGTGTTTGTATAGAAAT
TAATCATCCACCGCGCAGCC

B. Tbx2/3 cis-regulatory element P

AACACACAAGCCGGTAGCTCGTGCGAAGCTAGCGAGAAATCGACGGAGAGAGAGGGACAGAGGG
GAGAGAGAATGAGAGAGCGTTAGACTAGGGGTGATTATTGTAAGCAAAGTCTACACAATAATACAG
CTGGCCACTACTAGACGT*GTATCTGGGTGGCTAAACCCTCACATGTTCATAGGGATACAGACACCGC*
TATTTTGTGAAAGATCCCAAGTTTCTCGGTGGAACATTTACTATCTGCCATCCATCATAACACTTCACT
ATTATTCCTCCAGTTGGGCGACTGTGTACACCCTCTGTACTACTCCGCATATTTATTTTTTTCCATATTT

CAGGATATTTCAATCCGAGTGTTTCTTGGTTATCTTCTTGCATTTTACTGGATAACCTTAGAGCCTCTA
GCTCAAGCTTTGGTGGTTCGTCGAGCTCGCCGACATCGTCTTTCTCTATGAAACCGGCATCGAACGAC
CACCACACC

C. Dlx cis-regulatory element U

GATTTGAATTTGCCACACCATTAGATTTTAACTAAATGACAAAAAAGAAGAAAACAGATTAACCCAG
CTCTCTTGTATCTCGACGTTTAACGTTATTTTTTCTCATAATAGTAGTCATTCGACCAAATAATTCAAAT
TCTGGTCACAATAACACAATCAAACGCACCCATGCTAAACGCTTTACAAGTAACGGGGGTGGATTGC
TTTAGGAGTTTTTACAACATAAAAGGCAAAGTAAAGTGAAAGTCAGTTCAACTGTGGCTTTTATCGTT
GTGCTGTTGATAACAA**G**TTTTATTCTTGTGAAGAGCGTGTGTATATCGTTTAAATAGTCTGAGAGAG
AAGGGTGATGAAAGGCTGCGAGGGTGGCGAATGACCATCTTTGGCGCCGCTGGTATTTTCTTCTAG
TGGTCAGTCTTGTCCAGGCGGCTTTGTCTATGGCCTCGACCAGTTCTATTCTCAGCTTAGTCCCTCGC
ATGC

D. Dlx *cis*-regulatory element B

AATCTTTTGTCAGTGGGTTGTATAGTGGAAAGGGTTCGGTGACGCGACGAGGGTGGCCGTACCAGT
AGTATTTCAAAGCCTCACGTTGGTTTGATACCATCGCTAGCCGCGGCGACGAAGCCTCTAGTGTCCG
AGAAACAAAAAAACACATTTTCCGCCCGACGGACGTATGCTAATGCCATGACGTAACCATCAGCATT
TTGGACAAAAGCT

E. Dlx *cis*-regulatory element P

ATCGAAATATGATGAAGAGTAGTATTTTGAGGACATTAATGCTTCTGAGAGTCTGCGAGTAGTATTG
TTCCACATTTTGTTT*TCTCATATTAACTACATCGGAGGACTTTTGCGACAGAGCCTGAGGAGCAAACA*
GACCAAGTACAATTCTTTCTATGGGTCGTTTTTTTTTTTTTAATATCCATGATAGATCCTGGATCACTTT
TTTTTTATAAATAAAGTTGTCAGAATTTATTTGCAAATTCTCATCAAATATTTCCTATCCACTTTTAGTA
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ACAAGTTTTTGAGGATTGTAAGCGTACCATTCTTCGTTAATCAACCTGTTTTTTCTATCCTGCTTTGAT
GTATTCGTTTGACGTTGGAAAG

4. Experimental Procedures

Modeling the effect of a BMP and *tbx2/3* MO on a downstream gene.

Here we simulate the effect of BMP MO and tbx2/3 MO on the expression level of a downstream gene in indirect and direct (feed-forward) connectivity. In the indirect circuit connectivity (Fig. 4B) the BMP pathway activates Tbx2/3 directly through the phosphorylated transcription factor SMAD1/5/8 (pSMAD1/5/8) and Tbx2/3 directly activates the downstream gene TF that encodes an aboral ectoderm transcription factor. In the direct circuit connectivity the BMP pathways activates Tbx2/3 and TF directly through pSMAD1/5/8 and Tbx2/3 feeds-forward into TF (Fig. 4C). In both cases Tbx2/3 is activated by additional early input, A1, (e.g., HIF-1α) that initiates Tbx2/3 activation two hours before SMAD1/5/8 phosphorylation starts, in agreement with our perturbation data and the co-staining of Tbx2/3 and pSMAD1/5/8 (1). We assume that all inputs are additive ("OR" logic). In our simulation we use a set of delayed differential equation to describe the mRNA and protein levels of Tbx2/3, TF and pSMAD1/5/8. These equations are based on a kinetic model that was developed previously and described in (2-5). We used the following set of differential equations to describe the levels of Tbx2/3 and pSMAD1/5/8 (all the kinetic parameters are explained below):

$$(1)\ \frac{dmTbx2/3(t)}{dt} = \frac{1}{2}\left(I_{A1} + I_{\max}\left(1 - \exp\left(-\frac{k_{bpSMAD}Y_{pSMAD}(t-T_m)}{I_{\max}}\right)\right)\right) - k_{dm}mTbx2/3(t),$$

$$(2)\ \frac{dTbx2/3(t)}{dt} = k_t mTbx2/3(t) - k_{dP}Tbx2/3(t),$$

$$(3)\ Delay(t) = \begin{cases} 0, t < 120, \\ I_0, 120 < t. \end{cases}$$

$$(4)\ \frac{dSMAD(t)}{dt} = Delay(t) - k_{dm}SMAD(t),$$

$$(5)\ \frac{dpSMAD(t)}{dt} = k_p SMAD(t) - k_{dP}pSMAD(t).$$

Here *mTbx2/3* and *Tbx2/3* are the levels of Tbx2/3 mRNA and protein per cell respectively, *SMAD* and *pSMAD* are the levels of the SMAD1/5/8 and pSMAD1/5/8 per cell respectively, *Delay(t)* is a mathematical function that introduces the delay between Tbx2/3 transcriptional activation and SMAD phosphorylation, and the occupancy of the pSMAD1/5/8 binding site, $Y_{pSMAD,}$ is described by:

$$(6)\ Y_{pSMAD} = \frac{K_r pSMAD(t)}{D_n + K_r pSMAD(t)}.$$

The number of mRNA molecules for cell of the downstream transcription factor, *TF*, in the indirect circuitry, Fig. 4B, is described by:

$$(7)\ \frac{dmTF(t)}{dt} = I_{\max}\left(1-\exp\left(-\frac{k_{bTbx2/3}Y_{Tbx2/3}(t-T_m)}{I_{\max}}\right)\right)-k_{dm}mTF(t),$$

and in the direct feedforward connectivity, Fig. 4C is described by:

$$(8)\ \frac{dmTF(t)}{dt} = \frac{I_{\max}}{2}\left\{\left(1-\exp\left(-\frac{k_{bTbx2/3}Y_{Tbx2/3}(t-T_m)}{I_{\max}}\right)\right)+\left(1-\exp\left(-\frac{k_{bpSMAD}Y_{pSMAD}(t-T_m)}{I_{\max}}\right)\right)\right\}-k_{dm}mTF(t).$$

Here the occupancy of Tbx2/3 binding sites, $Y_{Tbx2/3}$, is similar to Eq. (6), replacing pSMAD level with Tbx2/3 protein level. The transcription initiation rates are the following: I_{A1}=0.15, I_{max}=11 mRNA/min. The rate of SMAD production is I_0=0.15 protein/min. The turnover rates are the following: $K_{dm} = 0.007$ min^{-1}, which means a half life of about one and a half hours and $K_{dP} = 0.005$ min^{-1}, which means a half life of about two hours. The transcriptional delay due to mRNA processing and export is $T_m=15$ min. The translation rate, $K_t = 2$ protein/(min × mRNA), SMAD phosphorylation rate $K_p = 2$ protein/(min × protein). The activation strengths are, $K_{bTbx2/3}$=1 and K_{bpSMAD}=5. The equilibrium constant $K_r=5\times10^4$ and the available genome $D_n= 7.2\times10^8$ bp, assuming that the open chromatin is about a 90% of the total sea urchin genome, which is 8×10^8 bp. For more information about the mathematical formalism, underlying assumptions and the values of the kinetic parameters please see (2-5). The results of this simulation are presented in Fig. 4.

References

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2. Ben-Tabou de-Leon S & Davidson EH (2009) Modeling the dynamics of transcriptional gene regulatory networks for animal development. *Dev Biol* 325(2):317-328.
3. Bolouri H & Davidson EH (2003) Transcriptional regulatory cascades in development: initial rates, not steady state, determine network kinetics. *Proc Natl Acad Sci U S A* 100(16):9371-9376.
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